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# Transgene expression and local tissue distribution of naked and polymer-condensed plasmid DNA after intradermal administration in mice

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ABSTRACT

DNA vaccination using cationic polymers as carriers has the potential to be a very powerful method of immunotherapy, but typical immune responses generated have been less than robust. To better understand the details of DNA vaccine delivery *in vivo*, we prepared polymer/DNA complexes using three structurally distinct cationic polymers and fluorescently labeled plasmid DNA and injected them intradermally into mice. We analyzed transgene expression (luciferase) and the local tissue distribution of the labeled plasmid at the injection site at various time points (from hours to days). Comparable numbers of luciferase expressing cells were observed in the skin of mice receiving naked plasmid or polyplexes one day after transfection. At day 4, however, the polyplexes appeared to result in more transfected skin cells than naked plasmid. Live animal imaging revealed that naked plasmid dispersed quickly in the skin of mice after injection and had a wider distribution than any of the three types of polyplexes. However, naked plasmid level dropped to below detection limit after 24 h, whereas polyplexes persisted for up to 2 weeks. The PEGylated polyplexes had a significantly wider distribution in the tissue than the nonPEGylated polyplexes. PEGylated polyplexes also distributed more broadly among dermal fibroblasts and allowed greater interaction with antigen-presenting cells (APCs) (dendritic cells and macrophages) starting at around 24 h post-injection. By day 4, co-localization of polyplexes with APCs was observed at the injection site regardless of polymer structure, whereas small amounts of polyplexes were found in the draining lymph nodes. These *in vivo* findings demonstrate the superior stability of PEGylated polyplexes in physiological milieu and provide important insight on how cationic polymers could be optimized for DNA vaccine delivery.



## 1. Introduction

The goal of vaccination is to manipulate the immune system into responding against specific antigens. Theoretically, this strategy can work both to treat ongoing infections and malignancies as well as prevent diseases by generating immunological memory. For successful immunization, antigen must be delivered to antigen-presenting cells (APCs), mainly dendritic cells (DCs) and macrophages, often along with an immunostimulatory adjuvant. These cells can then process and present the antigen and stimulate T and B cells in the lymph nodes and spleen [1–3].

Transfection of cells with antigen-encoding plasmid DNA will result in the expression of the protein antigen by those cells. This is an attractive method of vaccination due to the high stability of plasmid DNA formulations, the potential for long-term antigen production, and the capacity of generating both humoral and cellular immune responses to multiple epitopes [3–6]. Initial attempt of DNA vaccination involved the injection of naked plasmid DNA. However, though this can result in immune responses, the efficiency of this method of delivery needs improvement [4,5,7]. Various delivery vehicles, including viral particles, liposomes, and polymeric materials, have since been used to help protect the DNA and increase transfection [4,8]. Polymeric carriers have the potential to be a very effective means of delivering antigen-encoding DNA for immunization, because polymers can be easily modified and optimized to acquire a wide range of characteristics. Delivery of DNA vaccine with various polymer-based systems has shown improvement in both humoral and cellular immune responses, but overall immune responses have not been sufficiently robust [4,9].

Over the years a large number of polymer DNA carriers with a large variety of chemical structures have been developed, many of which have been investigated for DNA vaccine delivery. It is not clear, however, how to further improve upon current designs because little is known about the specific mechanisms of delivery *in vivo*, especially tissue and cellular fate of the DNA that precede immune response generation. There have been studies conducted to investigate the uptake and transfection of specific cell types using various naked DNA delivery systems in the muscle and skin [10–13]. For example, it was shown that fibroblasts, endothelial cells, and adipocytes appeared to be the primary cell types transfected after naked DNA delivery via electroporation in the skin [11], and that myocytes were the primary recipient of DNA

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after intramuscular injection [10]. More recently, van den Berg and others described the transfection of primarily keratinocytes after tattooing with PEGylated polyplexes [5]. These studies also reported some, if limited, DNA uptake by, or transfection of, APCs. However, exactly which cell types are involved in the interactions with polyplex-delivered DNA, and how those interactions may affect antigen presentation as well as the timeline and magnitude of immune responses, is not well understood. Furthermore, there is a lack of systematic understanding of the structure-function relationship of the polymer carriers in the context of *in vivo* administration. To this end, we prepared polyplexes of plasmid DNA and three cationic polymers with distinct chemical structures: branched polyethylenimine (PEI), linear poly(2-aminoethyl methacrylate) (PAEM), and diblock copolymer PEG-*b*-PAEM (Fig. 1). We injected these polyplexes into mice intradermally, and analyzed the transgene expression and local biodistribution of plasmid both macroscopically at the tissue level and microscopically at the cellular level, in comparison to injections of naked plasmid. We uncovered important differences in local tissue distribution between polyplexes and naked plasmid, and between PEGylated and non-PEGylated polyplexes. This information could be highly useful for improving the design of cationic polymer carriers for DNA vaccine.

## 2. Materials and methods

### 2.1. Chemicals and solvents

PEI (branched, 25 kDa) was obtained from Sigma. Monomethoxy-PEG (average $M_n$ of 5000) was from Aldrich and was used after vacuum drying at 80 °C for 2 h. Toluene (Aldrich) was dried by refluxing over sodium and distilled. The monomer *N*-(*tert*-butoxycarbonyl)aminoethyl methacrylate (*t*BAM) and the PEG macro-initiator for atom transfer radical polymerization (ATRP) was synthesized as described before [14–15]. Ethyl α-bromoisobutyrate, copper (I) chloride (CuCl) and 2,2′-dipyridyl (bPy) were purchased from Sigma. Other chemicals and solvents were purchased from Sigma and used without further purification.

### 2.2. Polymer synthesis

The ATRP of P*t*BAM followed a procedure modified from Tang et al. [16]. A glass two-neck flask was charged with *t*BAM, CuCl, bPy, and the system was degassed three times. Dried degassed toluene and ethyl α-bromoisobutyrate were added, and the mixture was heated at 80 °C for 8 h. The reaction was terminated by exposing the system to air. The reaction solution was then diluted by dichloromethane and passed through a basic aluminum oxide column to remove the copper complex. The resulting product was precipitated in hexane twice and dried in vacuum at room temperature for 2 days. To remove the Boc groups, 0.8 g of P*t*BAM was dissolved in 5 mL of trifluoroacetic acid and stirred for 2 h at room temperature. TFA was then removed by evaporation, and the oil residue was rinsed three times with diethyl ether. The resultant precipitate was collected by filtration, washed twice by diethyl ether, and dried overnight in vacuum. Afterwards, the polymers were washed with NaOH solution at pH 9.0, and immediately placed into dialysis tubing (MW cut-off 3500) and dialyzed against distilled water for 3 days. The final PAEM polymer was obtained by lyophilization.

PEG-*b*-PAEM diblock copolymer was synthesized as described in Tang et al [16] using a 5000-Da PEG block. The final polymer was washed by NaOH solution, dialyzed and lyophilized. The average values of $M_n$ for P*t*BAM and PEG-*b*-P*t*BAM were $3.37\times10^4$ and $3.96\times10^4$ with narrow distribution (PDI 1.16 and 1.20). Therefore, the average chain-length of the PAEM homopolymer (degree of polymerization, DP: 150) was the same as the PAEM segment in the PEG-*b*-PAEM diblock copolymer.

### 2.3. Polyplex preparation

Plasmid DNA encoding ovalbumin (pOVA, kindly provided by Dr. Chris Pennell) was purified from *E. coli* DH5α cells using an Endo-Free Plasmid Maxi plasmid prep kit (Qiagen) for tissue distribution studies. The plasmid was covalently labeled with Cy3 fluorophore using a Label IT nucleic acid labeling kit (Mirus) and purified according to manufacturer's instruction (including exhaustive dialysis to remove any free dye). Purified plasmid was verified to contain less than 0.6 EU/mg of endotoxin using the Pyrogent Gel Clot LAL assay kit (Lonza) and stored at −20 °C in sterile water. Polymer stocks were first diluted in 5% glucose, filter-sterilized, and stored in aliquots at −20 °C. To form polyplexes, polymer stocks were further diluted in sterile 5% glucose before DNA was added and samples were vortexed to mix. A typical batch of polyplexes was made with 12 μg of DNA and enough polymer for an N/P ratio of 8 in 36 μL total volume. Naked DNA was diluted to the same volume with 5% glucose. A luciferase plasmid (pCMV-LUC, endotoxin-free, Elim Biopharmaceuticals) was also used for *in vivo* gene expression experiments.

### 2.4. Polyplex stability in serum-containing medium

Fifteen microliters of polyplex solution containing 5 μg of Cy3-labeled plasmid DNA was added to another 15 μL of either 5% glucose or cell culture medium comprised of DMEM medium (1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (all cell medium components were from Gibco). Five microliters of the polyplexes solution was removed immediately after dilution and after a 1-h incubation at room temperature, placed onto a glass microscope slide, covered with a glass coverslip, and was observed under an Olympus IX70 inverted microscope equipped with a standard FITC/TRITC/DAPI filter set, a 20× objective lens, an Olympus DP72 camera, and CellSens software. To see if there was any free DNA present after polyplexes were formed and after incubation in 5% glucose and serum-containing medium, polyplex solutions were analyzed on a 0.7% agarose gel stained with ethidium bromide.

### 2.5. Injections

Hair was plucked from a small section of skin on the hind leg of 10- to 16-week old male C57BL/6 mice (Jackson Labs) to mark injection site, and polyplex solutions were injected intradermally through a 29-gauge needle. For transfection experiments, 40 μg of DNA complexed with polymers at N/P ratio of 8 was prepared as described above and was injected into each mouse in a total volume of 35 μL. For tissue distribution studies, 10 μg of DNA as polyplexes

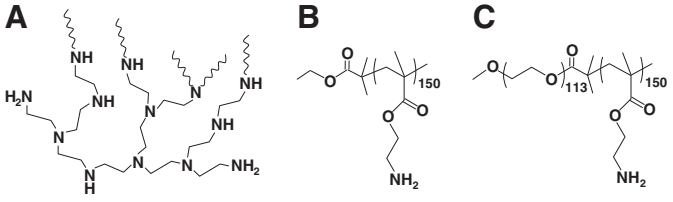


**Fig. 1.** Chemical structures of branched PEI (A), PAEM (B), and PEG-*b*-PAEM (C).